Curcumin Mediates Both Dilation and Constriction of Peripheral Arterioles via Adrenergic Receptors

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Curcumin has wound healing attributes mediated through a plethora of biological activities that in general are not ascribed to specific receptors. Recently, we have demonstrated that intravenous administration of curcumin limits burn injury progression in a rat model. As decreased microvascular perfusion is a central element of burn injury progression, we hypothesized that curcumin may induce vasodilation in peripheral arterioles, to improve perfusion. Using mucosal microcirculation as an *in situ* assay, cheek pouch tissue was exteriorized in anesthetized (phentobarbital 70 mg kg^{-1} intraperitoneal) male hamsters ($N=60$) to observe the terminal feed arterioles (~8 μm diameter) and the immediately upstream arcade arterioles (~20 μm). Curcumin (10^{-12}–10^{-4} mol l^{-1}) was applied dose-wise (micropipette, 60 seconds). Subnanomolar curcumin dilated, whereas micromolar doses constricted, the arterioles. For the terminal arteriole: vasodilation logEC$_{50}$ -10.3 ± 0.2, peak dilation $+39\pm1\%$; vasconstriction logEC$_{50}$ -8.0 ± 0.4, peak constriction $-14\pm2\%$. Simultaneous atropine (muscarinic antagonist) or PD142893 (endothelin antagonist) had no effect. Propranolol (β-adrenergic receptor (β-Ad) antagonist) enhanced constriction by removing the vasodilation response to curcumin. Phentolamine (α-adrenergic receptor (α-Ad) antagonist) enhanced dilation to curcumin by removing the vasoconstriction response. Thus, the curcumin vasomotor activity on microcirculation was α-Ad and β-Ad receptor-dependent and its net vasoactive effect was concentration- and time-dependent.

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INTRODUCTION

Curcuma longa (turmeric), an herb belonging to the ginger family, has wound healing properties attributable to a plethora of specific biologic activities that have not for the most part been ascribed to a known receptor (Aggarwal and Sung, 2009). Recently, we have shown that administration of curcumin to rats before (gastric gavage) or 1 hour after (intravenous) hot comb burns will limit injury progression (Singer *et al.*, 2007, 2011) in a manner unrelated to its antioxidant properties (Singer *et al.*, 2011). Alternatively, blood vessel occlusion by red blood cell plugging and thrombosis formation is found in the ischemic tissue surrounding a burn (Moritz, 1947; Regas and Ehrlich, 1992; Vo *et al.*, 1998) and may be why tissue necrosis extends from the primary burn site. As curcumin is vasoactive in large arteries from some tissues and species (Sasaki *et al.*, 2003; Gilani *et al.*, 2005; Xu *et al.*, 2007; Ahn *et al.*, 2009), we wondered whether some of the beneficial effect of curcumin was related to a vasomotor effect on the microvasculature.

Few studies have shown that curcumin is vasoactive; no previous report has investigated curcumin's action on the microcirculation. Studies in macrovessels typically tested micromolar levels of various curcuminoid preparations and found a decrease in tension development for artery rings from porcine coronary arteries, rat aorta, and rabbit basilar arteries (Sasaki *et al.*, 2003; Xu *et al.*, 2007; Ahn *et al.*, 2009), but not rabbit aorta (Gilani *et al.*, 2005); decreased tension is interpreted as dilation. From the limited literature, the mechanism by which curcumin decreases tension appears to be at most half nitric oxide (NO) mediated (half attributed to β-adrenergic receptor (β-Ad), Xu *et al.*, 2007), yet the vasoactivity appears to widely differ by tissue and species, or perhaps by curcuminoid preparation or extraction procedure. An important consideration from the cell culture literature is the well-documented cytotoxicity of curcumin, beginning at concentrations of 10^{-5} mol l^{-1} (Foresti *et al.*, 2005; Gilani *et al.*, 2005; Kunwar *et al.*, 2008). Thus, any response, or lack thereof, to curcumin in the micromolar range should be interpreted carefully.

In this study, we define the vasoactive response to curcumin in peripheral mucosal arterioles using the hamster cheek pouch *in situ* assay. The buccal mucosa of the hamster has a two-dimensional microcirculation, which greatly facilitates direct microscopic examination of vasoactive responses. In humans, both the buccal mucosa and cutaneous

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Abbreviations: cGMP, cyclic guanosine monophosphate; ET, endothelin; LNNA, N$^{\omega}$-nitro-L-arginine; NO, nitric oxide; α-Ad, α-adrenergic receptor; β-Ad, β-adrenergic receptor

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microcirculation consist of three-dimensional capillary loops, which arise from deeper feeding arterioles (Braverman, 1997; Scardina *et al.*, 2009). However, the diverse tissues display a similar vasoactive response capability (Gyorfi *et al.*, 1992; Oaklander and Siegel, 2005) to what is found for the hamster cheek pouch (Frame and Mabanta, 2007; Frame *et al.*, 2007; Georgi *et al.*, 2010), likely due to a similar vascular receptor distribution that includes adrenergic, muscarinic, and endothelin (ET) receptors (Hardman and Limbird, 2001). Thus, the hamster cheek pouch microcirculation was used as an *in situ* assay model system to facilitate direct investigation of possible curcumin vasoactivity on the microcirculation and, if any, of the receptor system by which it acts.

In this study, we hypothesized that ethanol extracted curcumin I (99% purity, Singer *et al.*, 2007) would induce a robust vasodilation in peripheral arterioles. In the hamster cheek pouch, we found that small arterioles do dilate to subnanomolar concentrations of curcumin. We also report that higher concentrations induce a robust constriction unrelated to vasoactivity of the vehicle ethanol. Both responses are recoverable. Further, the constrictor response is mediated by α-adrenergic (α-Ad) receptors, and the dilatory response is entirely mediated by β-Ad receptors and requires cyclic guanosine monophosphate (cGMP), yet, not all dilation appears to require NO formation.

RESULTS

Over the 60 seconds exposure time, low nanomolar and picomolar concentrations of curcumin induced a sustained dilation. At higher concentrations, initial vasodilation at 20 seconds was followed by vasoconstriction at 60 seconds. One experiment is shown in Figure 1 to illustrate the time and concentration dependence of this response. (Illustrative examples of control, dilated, and constricted arterioles are shown in Figure 1). Thus, we examined two separate concentration–response relationships: the early response at 20 seconds of exposure and the late response at 60 seconds of exposure (Figure 2a). Corresponding effective concentration at half-maximal response and maximal values are given in Supplementary Table S1 online. Peak dilation was significantly greater for terminal arterioles versus arcade arterioles.

Two important controls were performed to determine whether the biphasic nature of the vasomotor response to curcumin was attributable to vehicle (ethanol) or to a cytotoxic effect of curcumin over the course of the experiments. Although vehicle (ethanol) alone caused significant constriction at 0.1 and 1% (Figure 2b), only the highest concentration of ethanol (1%) yielded a constriction that could not be distinguished from constriction to curcumin. Furthermore, the initial vasodilation response cannot be explained as a response to vehicle, and therefore must be a response to curcumin itself. To determine whether vasoconstriction responses to curcumin were attributable to a cytotoxic effect, we demonstrated that both constrictor and dilatory (cGMP and cAMP mediated) responses were unchanged before versus after repeated exposure to curcumin (Supplementary Table S2 online). Thus, curcumin stimulated a recoverable dose- and time-dependent dilation/constriction response in hamster cheek pouch arterioles.

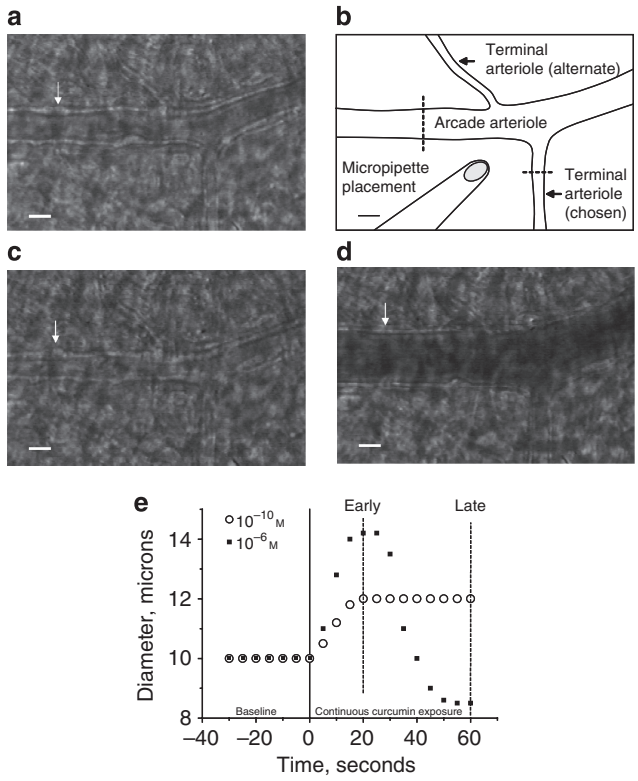

Figure 1. Arteriolar location and time dependence of diameter changes. Representative images of the arcade arteriole–terminal arteriole junction with micropipette placement for curcumin administration (schematic, **b**) in the basal control state (**a**), constricted with phenylephrine (**c**), and dilated with adenosine (**d**). Dashed lines in panel **b** indicate typical locations for diameter measurements. White arrow points to a bulge, which is a vascular smooth muscle cell in cross-section; especially in panel **c** the cells are seen as a ''string of pearls'' along the vascular wall. With dilation (**d**) the cells flatten and are not apparent. (**e**) Time course of the diameter change in response to continuous curcumin application (micropipette) in one terminal arteriole. Low-dose curcumin induced a sustained vasodilation, peaking by 20 seconds. Higher dose curcumin initiated dilation, peaking by 20 seconds, followed by a secondary constriction, peaking by 60 seconds. Data for concentration response analysis were thus taken at both the early (20 seconds) and late (60 seconds) time periods. Scale bar in **a**–**d** is 20 microns.

Adrenergic blockade suppressed the response to curcumin. Blockade was confirmed (Supplementary Table S3 online). Figure 3a shows that the dilation response to curcumin in the terminal arteriole was abolished by propranolol and the constriction response abolished by phentolamine. Propranolol and phentolamine applied together blocked all response to curcumin (Figure 3b). We eliminated the possibility that curcumin was initiating part or all of these vasoactive effects through stimulation of the sympathetic nerves causing norepinephrine release by showing that, although 6-hydroxy dopamine did significantly decrease baseline diameters (terminal arteriole, from 9.4 ± 0.8 to 11.2 ± 0.6 μm; arcade arteriole, from 19 ± 3 to 29 ± 4 μm), it did not remove the response to curcumin

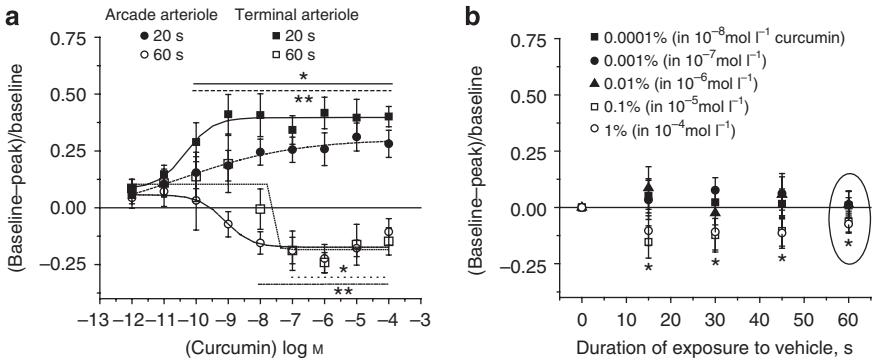

Figure 2. Arteriolar responses to curcumin or vehicle. Concentration–response relationship for curcumin + vehicle (**a**) or vehicle alone (**b**, ethanol) applied to arcade and terminal feed arterioles (Protocol 1). (**a**) The early response was obtained at 20 seconds and the late response was obtained at 60 seconds of continuous exposure to curcumin. The effective concentration at half-maximal response and maximal values are given in Supplementary Table S1 online. *, Terminal and **, arcade: individual responses differ from baseline, $P<0.05$. (**b**) Time course of the response to ethanol alone, showing the percent ethanol (in the corresponding curcumin solution). Terminal and arcade arterioles are combined. The response at 60 seconds (circled) corresponds to the late time point in panel **a** for all ethanol concentrations. *Differs from baseline for 0.1 and 1% ethanol only, $P<0.05$.

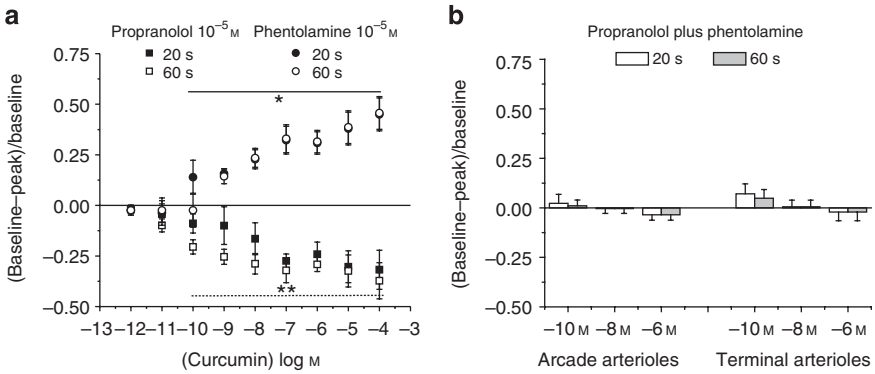

Figure 3. Propanolol, a β-adrenergic antagonist, inhibited curcumin-induced vasodilation, while phentolamine, an α-adrenergic antagonist inhibited curcumin-induced vasoconstriction. Shown are the early (20 seconds) and late (60 seconds) diameter changes in response to curcumin in the presence of 10^{-5} M propranolol or 10^{-5} M phentolamine separately (**a**) or together (**b**) (Protocol 2). Three concentrations of curcumin were tested in panel **b**: 10^{-10}, 10^{-8}, and 10^{-6} M. Responses are shown only for the terminal arteriole in panel **a**, and for both arcade and terminal feed arterioles in panel **b**. The effective concentration at half-maximal response and maximal values for both the terminal arteriole and arcading arteriole are given in Supplementary Table S1 online. *, Phentolamine and **, propranolol: individual responses differ from baseline, $P<0.05$.

(Supplementary Table S1 online). Thus, the response to curcumin was prevented with total adrenergic blockade, and not by blocking sympathetic nerves, suggesting a direct action on the vascular wall by curcumin.

Atropine (muscarinic antagonist) or PD142893 (ET antagonist) each diminished the maximal dilation and enhanced the maximal constriction to curcumin (Supplementary Table S1 online), yet had no effect on baseline diameters. Blockade was confirmed (Supplementary Table S3 online). Although muscarinic or ET receptor-mediated actions may be involved in modulating the response to curcumin, they are not required.

The β-Ad receptors may be present on endothelial cells, where they induce a NO, cGMP-mediated dilation. Alternatively, or in addition, β-Ad receptors may be present on vascular smooth muscle cells where they induce a cAMP-mediated dilation (Hardman and Limbird, 2001). The NO dependence of dilation to curcumin was tested by blocking endogenous NO formation with N^{ω}-nitro-L-arginine (LNNA). With 10^{-5} M LNNA, dilation to curcumin was attenuated to an equivalent extent in both the terminal feed and arcade arterioles; however, a significant dilation remained at the 10^{-12} to 10^{-10} M curcumin concentrations (Figure 4a). At higher 10^{-4} M LNNA, significant residual dilation remained for the lower concentrations of curcumin (Figure 4b). Blockade was confirmed (Supplementary Table S3 online). These results suggested that dilation was mediated by NO at least in the nanomolar and micromolar range, but other mechanism(s) contributed to the microcirculation response in the picomolar range, perhaps consistent with low concentrations acting through an alternate G-protein system, or the high concentrations preferably acting directly on the downstream kinase elements (Hardman and Limbird, 2001; Sun *et al.*, 2007).

Next, we blocked cGMP and cAMP directly, using the Rp isomers, Rp-8-br-cGMPS and Rp-8-br-cAMPS (Figure 5).

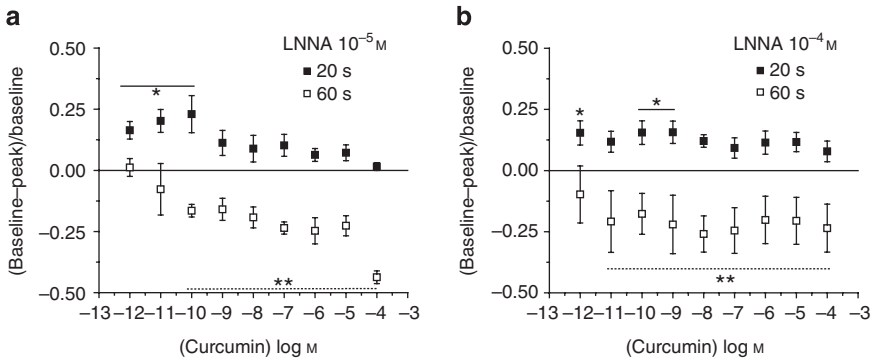

Figure 4. N$^{\omega}$-nitro-L-arginine (LNNA) partially inhibits curcumin-induced vasodilation. Shown are the early (20 seconds) and late (60 seconds) diameter changes in response to curcumin in the presence of LNNA (**a**, 10^{-5} M; **b**, 10^{-4} M; Protocol 2). Responses are shown for the terminal arteriole only. The effective concentration at half-maximal response and maximal values for both the terminal arteriole and arcading arteriole are given in Supplementary Table S1 online. *, Early and **, late: individual responses differ from baseline, $P<0.05$.

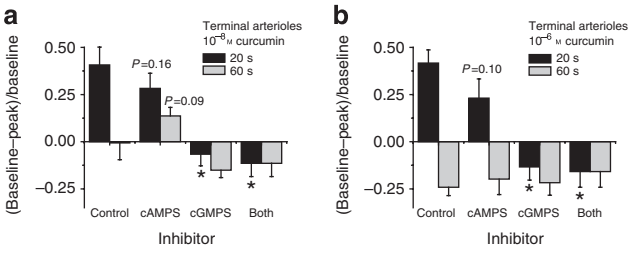

Figure 5. Inhibition of cyclic guanosine monophosphate (cGMP) abolished curcumin-induced vasodilation. Shown are the early (20 seconds) and late (60 seconds) diameter changes in response to curcumin in the presence of 10^{-5} M Rp isomers to block cGMP (Rp-8-br-cGMPS) or cAMP (Rp-8-br-cAMPS; Protocol 3). Two concentrations of curcumin were tested: (**a**) 10^{-8} M and (**b**) 10^{-6} M. Responses are shown from both the terminal arterioles (**a** and **b**) and arcade arterioles (**c** and **d**). *Differs from control for the associated early versus late response.

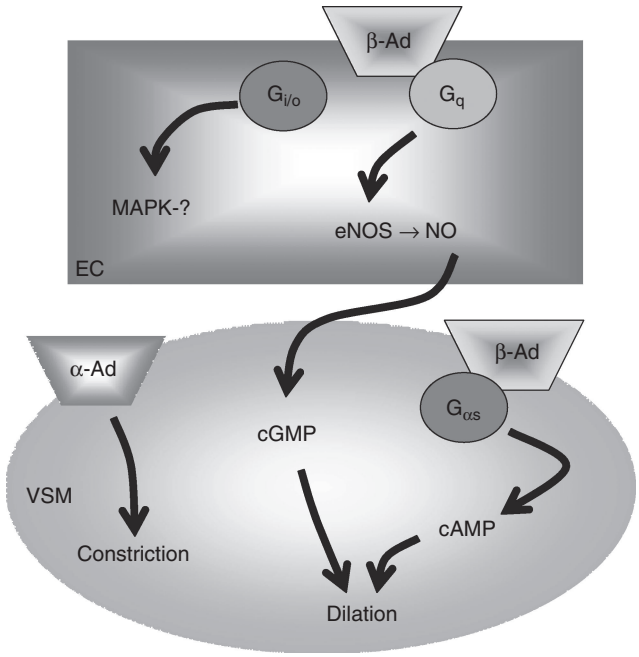

Figure 6. Schematic of hypothesized mechanism of vasoaction for purified curcumin I in the peripheral microcirculation for terminal (nutritive) arterioles or arcade arterioles that feed them. Curcumin induces vasoconstriction that requires α-Ad, and vasodilation that requires β-Ad. EC-dependent dilation occurring through endothelial nitric oxide synthase (eNOS), nitric oxide (NO), and cyclic guanosine monophosphate (cGMP) accounts for most of the dilation; we hypothesize that this is linked to the receptor G-protein subunit Gq and independent of Gi or mitogen-activating protein kinase (MAPK) activity. VSM-dependent dilation occurring through cAMP accounts for a smaller portion of the dilation, significant in only the arcade arterioles; based on the data presented here, we hypothesize that this is linked to the G-protein subunit $G\alpha_s$. α-Ad, α-adrenoceptor (VSM constriction); β-Ad, β-adrenoceptor (EC or VSM dilation); EC, endothelial cell; VSM, vascular smooth muscle cell.

Blockade was confirmed (Supplementary Table S3 online). Blocking cAMP significantly suppressed dilation to curcumin at 10^{-6} mol l^{-1} for the arcade, but not terminal arterioles (Figure 5). Furthermore, in arcade arterioles the constrictor response to 10^{-8} mol l^{-1} curcumin was attenuated with Rp-8-br-cAMPS. Blocking cGMP prevented all dilation to curcumin in both the terminal and arcade arterioles (Figure 5). Thus, the dilation to curcumin appears to require cGMP for both classes of vessels, yet a significant role for cAMP-mediated dilation is suggested for the larger arcade arterioles.

DISCUSSION

Here we show that picomolar to low nanomolar concentrations of curcumin I induced a sustained vasodilation in hamster cheek pouch peripheral arterioles that was dependent on β-Ad receptor activity (Figure 6). Dilation was consistent with the response to curcumin observed for coronary arteries (Xu *et al.*, 2007). However, curcumin in the micromolar range induced sustained microvasculature constriction that was dependent on α-Ad receptor activity. In contrast, other studies using large arteries demonstrated that micromolar concentrations of curcumin induced decrease tension that was interpreted as vasodilation (Sasaki *et al.*, 2003; Gilani *et al.*, 2005; Xu *et al.*, 2007; Ahn *et al.*, 2009). Persistent vasoconstriction in vascular tissue to

alcohol-extracted curcumin I is to our knowledge a previously unreported finding, which cannot be attributed to transient responses to vehicle alone.

Propranolol blocked all dilation to curcumin. In this tissue, β2-Ad are present (Koo, 1984), but it was unknown whether they resided on endothelial and/or vascular smooth muscle cell types. While α2-Ad can induce dilation in some tissues and species (Crassous *et al.*, 2009), their role was not investigated secondarily due to our conclusive findings with propranolol. We investigated the cell type pharmacologically with LNNA (to block endothelial-dependent dilation) and then with Rp isomers. With LNNA, dilation remained significant for the picomolar concentrations of curcumin, suggesting that the NO–cGMP pathway was required. Supporting this possibility, abrogating cGMP formation directly, prevented significant dilation. We did not investigate whether curcumin was acting on a kinase downstream of the β2-Ad receptor (for example, mitogen-activating protein kinase, extracellular signal-regulated kinases 1/2, and have not ruled out an inverse agonism effect (Hardman and Limbird, 2001; Woo *et al.*, 2005; Sun, McGarrigle and Huang, 2007). Our finding is strikingly different from that of Xu *et al.* (2007), who reported that only half of the dilation to (synthesized) curcumin I in porcine coronary arteries is blocked with propranolol.

Dilation to curcumin was greater for the terminal arterioles than the arcade arterioles. This is consistent with findings in the microvasculature of the rat cremaster, in which the terminal arterioles exhibited a more robust dilation through cGMP than did the arcade arterioles (Frame *et al.*, 2002), and may underlie a fundamental difference in the responses for these two classes of arterioles. The arcade arterioles were sensitive to cAMP blockade, suggesting a significant role for vascular smooth muscle cell β-Ad receptors in mediating the dilation to curcumin. We speculate therefore that the residual dilation at low curcumin concentrations with LNNA is through the vascular smooth muscle β-Ad, the magnitude of which differs by vessel class in this tissue.

We investigated alternate mechanisms that could explain a residual dilation with LNNA, and eliminated the possibility of an indirect process for curcumin-induced dilation through sympathetic nerve release of norepinephrine (6-hydroxy dopamine data). Finally, based on the literature, we do not believe that an alternate mechanism is responsible for these effects. For example, curcumin has a host of actions on cellular processes (Aggarwal and Sung, 2009), including induction of hemeoxygenase-1 that produces carbon monoxide (CO, Ryter *et al.*, 2005). CO targets guanylyl cyclase to produce cGMP, causing vasodilation (Samora *et al.*, 2010). However, the several hour timeline of curcumin-induced expression of hemeoxygenase-1 does not fit our vasoactive responses, which were acute and immediate.

Comparing our study to the paucity of literature examining vasoactive responses to curcumin, we speculate that three factors are important to account for differences in response: the curcuminoid itself; the toxic nature of micromolar curcumin; and the vessel size and species tested. From the literature, constriction to curcumin (seen as increased force development in arterial rings) has only been shown for the polysaccharide extracted component of crude curcumin (Sasaki *et al.*, 2003) or for non-vascular tissue (Gilani *et al.*, 2005). The highly purified curcumin preparation that we used was solvent extracted followed by high-pressure liquid chromatography verification of curcumin I (>99%; Singer *et al.*, 2007), and thus constriction in our hands is not due to other curcuminoids. Previous findings by others using crude, solvent-extracted curcumin I, or synthesized curcumin I, indicated decreased tension (interpreted as vasodilation) in artery rings in the micromolar range, without evidence of increased tension (vasoconstriction; Sasaki *et al.*, 2003; Gilani *et al.*, 2005; Xu *et al.*, 2007; Ahn *et al.*, 2009). The authors' interpretation of their data was that the continued decrease tension observed with increasing concentrations of curcumin was attributable to β-adrenergic receptors, part of which was prevented by propranolol (Xu *et al.*, 2007). However, a return to control tension after curcumin removal was not shown for these studies, and in most cases the total time of curcumin exposure was not given (Foresti *et al.*, 2005; Gilani *et al.*, 2005; Kunwar *et al.*, 2008). It is therefore unknown whether the total change in tension was an active process in these macrovessels, or whether high curcumin concentrations were cytotoxic over their incubation periods. In contrast, we directly observed small arterioles *in situ* with brief exposures and verified return to baseline. Curcumin induced a robust, sustained vasodilation at picomolar to low nanomolar levels that was completely recoverable with repeated 60 seconds exposures, as evidenced by no change in dilatory and constrictor capability before versus after curcumin. To our knowledge the vasoactive response of curcumin in peripheral microvessels is previously unreported.

In conclusion, in the hamster cheek pouch model, curcumin is a potent vasodilator in the subnanomolar concentration range, shifting to constriction in the micromolar range. Both responses can be attributed to adrenergic receptors directly. Upon examination of the molecular structure of curcumin (Aggarwal and Sung, 2009) and its close similarity to norepinephrine, and α-Ad or β-Ad agonists and antagonists (Hardman and Limbird, 2001), our finding that curcumin functions through these receptors is not surprising. Expanding our interpretation of these findings to other microvascular tissue beds, curcumin is capable of inducing either constriction or dilation, and dilation can be endothelium-dependent or -independent. The precise adrenergic receptor distribution in each tissue will determine vasoactive responses to curcumin.

Importantly, the clinical relevance of this finding is directly related to our ongoing work that shows the beneficial effect of nanomolar curcumin in ameliorating thermal burn progression (Singer *et al.*, 2007, 2011). In specific, curcumin administered intravenously 1 and 24 hours after thermal injury reduced burn injury progression (Singer *et al.*, 2011). A potential mechanism by which this occurs is by dilation of the blood vessels in the immediate vicinity of the burn, in the zone of potential ischemia.

MATERIALS AND METHODS

Animal model

With approval by the State University of New York at Stony Brook's Institutional Animal Care and Use Committee, male adult hamsters (120 ± 12 g, 116 ± 34 days, $N = 60$) were anesthetized with pentobarbital sodium ($70\,mg\,kg^{-1}$ intraperitoneal) and tracheostomized. The left cheek pouch tissue was exteriorized and dissected for intravital microscopy observations of terminal arterioles coursing through loose areolar connective tissue on the adventitial side of the pouch. The thick keratinocyte layer that is normally open to the buccal cavity was placed face down on the dissection board and not directly observed. For additional histological orientation, please refer to Figure 1 in reference Murray *et al.* (2010). The control tissue bath solution was bicarbonate-buffered saline (in $mmol\,l^{-1}$: 132 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, and 20 $NaHCO_3$ (equilibrated with gas containing 5% CO_2-95% N_2 gas, pH 7.4 at 34 °C, without proteins added; Frame and Mabanta, 2007; Frame *et al.*, 2007; Georgi *et al.*, 2010), which was flowed continuously over the tissue at $5\,ml\,min^{-1}$. Adenosine ($10^{-4}\,mol\,l^{-1}$) and phenylephrine ($10^{-4}\,mol\,l^{-1}$) were dripped onto the tissue and used to confirm dilator and constrictor tone, respectively. Thirty minutes later, microvascular responses (diameter change) were obtained according to Protocols 1–3, below.

Arteriolar networks

The hamster cheek pouch is a 400–500 μm thick tissue composed of repeating terminal arteriolar networks arising from arcading arterioles. One terminal arteriolar network consists of a terminal feed arteriole providing nutrient flow to 3–5 terminal branches (Frame and Mabanta, 2007; Frame *et al.*, 2007; Georgi *et al.*, 2010). By definition the terminal branches only feed capillaries. The cheek pouch tissue is composed of both a muscular and mucosal region. The mucosal region is innervated by only sensory nerves, whereas the muscular region (retractor muscle) is innervated by sensory, motor, and autonomic nerves (Grasby *et al.*, 1999); here, we examined the mucosal region only. In this study, curcumin was applied through micropipette to the entrance to the terminal network as the terminal arteriole arose from the arcade, thus defining the response to curcumin for two classes of blood vessels: conduit arcade arterioles and nutritive terminal arterioles (directly feeding capillaries).

Micropipette drug application

Micropipettes were made (Pipette Puller, Model 740 David Kopf Instruments Tujunga, CA) with 4–8 μm diameter tip openings over a distance of 100–200 μm. They were backfilled with test agents plus a flow tracer, $10^{-5}\,mol\,l^{-1}$ FITC–BSA, and positioned ~25 μm from the vessel wall. Controlled delivery of test agents was achieved using a pressure ejection system (MPPI-2, Applied Scientific Instrumentation, Eugene, OR), in which the balance pressure was set so that no FITC dye ejection was visible in the holding position, and the ejection pressure was the minimum that provided a steady outflow of FITC (typically 0.2 psi). Owing to the position of the micropipette relative to the test arteriole and the constantly flowing superfusate, micropipette contents were washed over the test arteriole and then immediately removed from the observation site. Using this technique, the volume ejected in a typical exposure is on the order of picoliters, and the concentration of test agents at the wall of the vessel is estimated to be half of the concentration in the pipette (Georgi *et al.*, 2010). Micropipette drug exposure was preceded by a 30-second baseline, and the test agent was applied for a prescribed time, as noted for individual protocols.

Protocol 1—locally applied curcumin

Curcumin obtained from Chromadex (Irvine, CA) was previously tested for purity; this ethanol extraction process followed by preparative high-pressure liquid chromatography yields curcumin I (>99%) as demonstrated by mass spectroscopy (Singer *et al.*, 2011). The purified curcumin ($10^{-2}\,mol\,l^{-1}$) was stored at −80 °C in ethanol until used, and then diluted in control suffusate (10^{-12}–$10^{-4}\,mol\,l^{-1}$, $n = 7$). In each animal, two to three networks were tested (minimum of 500 μm apart), performing the complete concentration–response at each site. We have previously shown that this distance assures independent observations in this tissue (Frame and Mabanta, 2007; Frame *et al.*, 2007; Georgi *et al.*, 2010); with curcumin, likewise, there was no difference in responses between sites. The highest dose of curcumin tested ($10^{-4}\,mol\,l^{-1}$) contained 1% ethanol. An ethanol dose–response was repeated here using 0.0001–1% ethanol, encompassing the range of 10^{-8}–$10^{-4}\,mol\,l^{-1}$ curcumin ($n = 3$). Curcumin, or ethanol alone, was applied for 60 seconds through micropipette to the junction where the arteriolar terminal feed arose from the arcade, exposing both vessel segments.

Protocol 2—suffusate applied antagonists

Only one antagonist was tested per animal, and two to three sites that were more than 500 microns apart were tested per animal. Phentolamine (α-Ad receptor antagonist, $10^{-5}\,mol\,l^{-1}$, $n = 6$), propranolol (β-Ad receptor antagonist, $10^{-5}\,mol\,l^{-1}$, $n = 6$), atropine (muscarinic receptor antagonist, $10^{-7}\,mol\,l^{-1}$, $n = 7$), or LNNA (LNNA, nitric oxide synthase antagonist $10^{-5}\,mol\,l^{-1}$, $n = 5$; and $10^{-4}\,mol\,l^{-1}$, $n = 7$) were added to the flowing control suffusate for 5 minutes before and then continuously while Protocol 1 was performed. In five additional animals, phentolamine and propranolol were applied together. Blockade was confirmed with phenylephrine (α-Ad receptor agonist, $10^{-5}\,mol\,l^{-1}$), isoproterenol (Ad receptor agonist, $10^{-5}\,mol\,l^{-1}$), acetylcholine (muscarinic receptor agonist, $10^{-4}\,mol\,l^{-1}$), and nitroprusside (cGMP-mediated dilation agonist, $10^{-4}\,mol\,l^{-1}$).

The sympathetic nerve toxin, 6-hydroxy dopamine ($10^{-3}\,mol\,l^{-1}$, $n = 5$) was applied to a stationary tissue bath. Suffusate flow was stopped, and bone wax was used to create a pool encircling the cheek pouch tissue. The neurotoxin was added to the pool for 20 minutes. Then, control suffusate flow was returned, and Protocol 1 was performed.

Protocol 3—micropipette applied antagonists

Only one antagonist was tested per animal, and two to three sites were tested per animal. PD142893 (ET receptor A and B antagonist, $10^{-5}\,mol\,l^{-1}$, $n = 4$) was applied to the observation site through micropipette for 5 minutes before and then continuously during curcumin exposure. Curcumin (10^{-12}–$10^{-4}\,mol\,l^{-1}$) was applied in increasing concentrations, as per Protocol 1. Rp-8Br-cGMPS (cGMP antagonist, $10^{-4}\,mol\,l^{-1}$) or Rp-8Br-cAMPS (cAMP antagonist, $10^{-4}\,mol\,l^{-1}$), separately and together at separate sites within the same animal ($n = 5$), was applied to the observation site through micropipette for 5 minutes before and then continuously during curcumin exposure to only 10^{-8} or $10^{-6}\,mol\,l^{-1}$ curcumin.

Blockade was confirmed with nitroprusside (cGMP-mediated dilation agonist, 10^{-4} mol l^{-1}) and adenosine (cAMP-mediated dilation agonist, 10^{-4} mol l^{-1}).

Microscopy and videorecording

The vasculature was observed using a modified Nikon (Tokyo, Japan) upright microscope with infinity optics. Data were obtained using a Nikon ×50 LWD objective (NA 0.45) imaged with a Gen/Sys intensifier and charge coupled device 72s (Dage MTI, Indianapolis, IN) camera, and recorded with a Panasonic AG-7350 SVHS (Secaucus, NJ) videorecorder. Diameter was measured off-line using a videocaliper system (FOR-A, Fort Lee, NJ) calibrated with a scale micrometer.

Statistical analysis

Diameter changes were calculated as the fractional change from baseline, ((baseline–peak)/baseline). The fractional change is reported to facilitate comparison of two sizes of arterioles. The level of dilatory and constrictor tone was similar for each class of arteriole. Concentration–response relationships were analyzed by fitting a sigmoid-shaped curve to the mean values and obtaining the effective concentration at half-maximal response and maximal values (Origin Laboratories Software (Northampton, MA), weighted by standard deviations). Comparisons were made between treatments using unpaired *t*-tests; when comparisons involved multiple sequential treatments (for example, doses), analysis of variance for repeated measures was used. All experimental design employed a β-probability function of 0.6, *a priori*, and a statistical significance, α-probability function of 0.05, *post hoc* (Snedecor and Cochran, 1974).

CONFLICT OF INTEREST

MD Frame, RA Clark, and AJ Singer have a patent pending for ''use of curcumin in thermal burns that identifies the mechanism of action by which curcumin ameliorates thermal burn injury progression''. Title: *Curcumin controls blood flow through adrenergic receptors*.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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